JOURNAL OF VIROLOGY, Nov. 2011, p. 11139–11145 Vol. 85, No. 21
0022-538X/11/$12.00 doi:10.1128/JVI.00169-11

Antimycotic-Antibiotic Amphotericin B Promotes Influenza Virus Replication in Cell Culture▽

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Received 25 January 2011/Accepted 2 August 2011

In general, antibiotics are not rated as substances that inhibit or support influenza virus replication. We describe here the enhancing effect of the polyene antibiotic amphotericin B (AmB) on influenza virus growth in Vero cells. We show that isolation rates of influenza A and B viruses from clinical samples can be dramatically enhanced by adding AmB to the culture medium. We demonstrate that AmB promotes the viral uptake and endocytic processing of the virus particles. This effect is specific for Vero and human nasal epithelial cells and was not observed in Madin-Darby canine kidney cells. The effect of AmB was subtype specific and more prominent for human seasonal influenza strains but absent for H5N1 human viruses. The AmB-enhancing effect seemed to be solely due to the viral hemagglutinin function. Our results indicate that the use of AmB may facilitate influenza virus isolation and production in Vero cells.

Until recently, influenza virus isolation from clinical samples and vaccine manufacture was almost entirely based on the infection of 9- to 11-day-old embryonated chicken eggs (CE). However, CE as a production substrate have serious restrictions because of the limited availability of high-quality eggs, especially in the case of an impending pandemic. In addition, some of the human strains of influenza virus do not replicate in eggs and require adaptation passages and/or a reassortment with a well-adjusted donor virus for improved growth. Moreover, the cultivation of human-derived influenza viruses in a host such as CE might lead to the selection of host range mutant variants that are characterized by structural changes in the hemagglutinin (HA) molecule, which, in turn, might have a negative effect on receptor specificity and the immunogenicity of egg-derived vaccines (1, 9, 25, 28).

Alternative approaches involving the use of accredited continuous cell lines have been pursued by several vaccine manufacturers in recent years. Madin-Darby canine kidney (MDCK), African green monkey kidney (Vero), or PER.C6 cells have been used successfully to produce influenza viruses (3, 13, 23, 32).

Vero cells are well characterized and widely accepted by regulatory authorities. They have been used to produce human virus vaccines, including those against poliomyelitis and rabies, for more than 30 years. Recently, they were also approved for use in vaccines against smallpox, rotavirus infection (7), and influenza (3). Increased yields of influenza virus in Vero cells could be achieved by implementing a serum-free medium cultivation (17) and multiple additions of trypsin to the medium (18).

The primate origin of Vero cells may have a positive impact on the retention of the biological properties of human influenza viruses. Compared to MDCK and egg-derived isolates, Vero-derived influenza virus strains proved to be more similar to human original viruses in terms of their receptor specificity and glycosylation pattern (21, 26). These characteristics would suggest the use of Vero cells for virus isolation and surveillance purposes, but despite all advantages, the isolation rates obtained in Vero cells still tend to be lower than those in MDCK cells.

We show here that the sensitivity of Vero cells to influenza virus infection can be dramatically improved by supplementing with the polyene antimycotic-antibiotic amphotericin B (AmB). We found that AmB is able to promote the initial steps of viral infection, including the enhancement of viral endocytosis and fusion with endosomal membranes. Improved infectivity in Vero cells resulted in better viral recovery from clinical samples.

MATERIALS AND METHODS

Reagents. The antibiotic-antimycotic mix was obtained from Sigma-Aldrich. AmB was used in its solubilized form (ca. 45% AmB, 35% sodium deoxycholate, and the rest sodium phosphate and chloride). It was obtained in either liquid (Fungizone; Sigma-Aldrich) or pulverized (AmB BMS; Bristol-Myers Squibb) form.

Cells. The Vero (African green monkey kidney) cells were obtained from the European Collection of Cell Cultures (ECACC; no. 88020401) or the American Type Culture Collection (ATCC; CCL-81) and were cultivated in serum-free conditions using OptiPRO SFM supplemented with 4 mM L-glutamine (SFM; both from Gibco/Invitrogen). The MDCK cells were obtained from ATCC (CCL-34) and cultured in ATCC-formulated Eagle minimum essential medium (Gibco/Invitrogen), supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen). Human nasal epithelial (HNE) cells (primary culture) were obtained from Provitro, Germany, and were cultured according to the manufacturer's instructions.

Human bronchial epithelial (16HBE14o$^-$) cells were kindly provided by J. Seipelt (Vienna, Austria). The cells were cultivated in minimal essential medium (MEM; Gibco/Invitrogen) supplemented with 10% FBS and 2 mM L-glutamine, and cell culture flasks were coated (the coating mix consisted of Ham F-12

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▽ Published ahead of print on 17 August 2011.

medium [Gibco/Invitrogen] supplemented with 10 μg of bovine serum albumin [BSA; Sigma-Aldrich]/ml, 30 μg of bovine collagen type I [Promocell]/ml, and 10 μg of fibronectin [BD Pharmingen]/ml).

A549 (human lung carcinoma) cells were obtained from ATCC (CCL-185). The cells were propagated in ATCC-formulated F-12K medium (ATCC) supplemented with 10% FBS.

Viruses. Clinical specimens were kindly provided by the Institute of Virology, St. Petersburg, Russia (H3N2, vH1N1), and the Institute of Virology, Medical University Vienna, Austria (H3N2, B). Additional swab material was obtained from the National Influenza Center for the World Health Organization (WHO), Laboratoire de Virologie, Lyon, France (H3N2, H1N1). The thawed original material was isolated on Vero or MDCK cells in SFM supplemented with 5 μg of trypsin (Sigma-Aldrich) and 25 μg of gentamicin (Gibco/Invitrogen)/ml. AmB (250 ng/ml) was added as indicated. After incubation for 5 to 7 days at 34°C and 5% CO_2, the virus supernatants were passaged by transferring 50 μl to fresh subconfluent cell monolayers.

All ΔNS1 reassortant viruses were obtained by reverse genetics solely on Vero cells (10, 14, 27, 34). The internal segments of influenza A viruses were derived from a modified IVR-116 vaccine strain (WHO) containing a deletion of the NS1 gene. They originate from A/Puerto Rico/8/34 (PA, PB2, NP, M, and NS) and A/Texas/1/77 (PB1). The H5N1 ΔNS1 reassortants (H5N1 and H5N1-K58I) inherited the HA from the H5N1 A/Vietnam/1203/04 strain (A/VN/1203/04) with a modified cleavage site (27). For the H5N1-K58I, a single HA2 mutation was introduced by site-directed mutagenesis (Stratagene) (19). The 7:1 reassortant viruses (H5 and vH1) were constructed by solely replacing the HA of the modified IVR-116 virus. The HAs and neuraminidases (NAs) of the other influenza A ΔNS1 virus constructs were of the following origins: A/New Caledonia/20/99 (H1N1), A/California/7/2009 (vH1N1), and A/Brisbane/10/2007 (H3N2). The influenza B ΔNS1 virus inherited the HA and NA from B/Florida/4/2006 and the internal segments from B/Thüringen/2/2006 with a modified NS segment (ΔNS1) (35). Virus stocks were prepared by infection of 70 to 90% subconfluent monolayers of Vero cells at multiplicities of infection (MOIs) between 0.05 and 0.001. SFM supplemented with 5 μg of trypsin/ml and optionally 250 ng of AmB/ml was used. Cultures were propagated at 37 or 34°C (influenza B virus only) and 5% CO_2. A/Puerto Rico/8/34(H1N1) (PR8) virus was cultivated in embryonated chicken eggs.

Virus infectivity assay. Vero cells were infected (MOI of 2 or 5) and grown in the presence or absence of 250 ng of AmB/ml. No trypsin was added to the SFM to assure a single-cycle replication. After 18 h of incubation at 37°C (influenza A virus) or 34°C (influenza B virus) and 5% CO_2, the supernatant was removed. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.2% Triton X-100 (Merck) in Dulbecco modified phosphate-buffered saline (D-PBS), and blocked (the blocking buffer consisted of D-PBS with 0.1% Tween 20 [Merck] and 1% BSA [PAA]). For the detection of influenza A virus-infected cells, test plates were incubated with a mouse anti-NP monoclonal antibody mix (Chemicon MAb 8251; dilution 1:1,000 in blocking buffer), followed by a secondary goat anti-mouse IgG/Alexa Fluor 488 antibody (Invitrogen; dilution 1:1,000 in blocking buffer). For influenza B virus, a mouse anti-influenza B virus MAb (Santa Cruz; dilution 1:500 in blocking buffer) was used as the primary antibody. Microscopic examination was performed with an Olympus CK X41 microscope with U-RFLT 50-200 UV lamp and attached Olympus camera system E330. Pictures were taken at ×10 magnification.

Virus titration. Infectious virus titers in 50% tissue culture infectious doses ($TCID_{50}$)/ml were determined in Vero cells seeded at a density of 1.5×10^4/well the day before infection. Tenfold virus dilutions were prepared. After a defined incubation period, the wells were examined microscopically and scored as infected or noninfected by determining the presence or absence of a cytopathic effect. The virus titer was calculated according to the method of Reed and Muench (24).

Flow cytometry. Cells were infected at an MOI of 4 in the absence of trypsin to assure single-cycle replication. AmB was added at 250 ng/ml as indicated.

At 4 h postinfection (p.i.) the cells were trypsinized and washed. Next, 10^6 cells were fixed and permeabilized by using a Cytofix/Cytoperm Plus kit (BD Biosciences) according to the manufacturer's instructions. The cells were incubated with fluorescein isothiocyanate (FITC)-labeled mouse anti-NP and anti-matrix (M) antibodies (reagent A, Imagen Influenza detection kit; Oxoid) or isotype-matched negative controls (FITC-labeled mouse IgG1 and IgG2a; Sigma-Aldrich). Afterward, the cellular fluorescence was analyzed in an EPICS XL-MCL flow cytometer using EXPO 32 software (Coulter Immunotech). A total of 10,000 events were acquired for each sample, and the percentage of positive cells was reported.

Electron microscopy (EM). The cells were inoculated at an MOI of 30. The infected cells were incubated for 1 h at 4°C. Extracellular virus was removed by washing and medium (SFM with 2 μg of TPCK [tosyl phenylalanyl chloromethyl ketone] trypsin/ml), containing either 500 ng of AmB/ml or no AmB, was added. The cell monolayers were incubated at 37°C and 6% CO_2. After the indicated time, the cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The cells were detached, and the pellets were washed twice with 0.1 M cacodylate buffer and postfixed in osmium tetroxide (1% in 0.1 M cacodylate buffer). The cells were then dehydrated and embedded in epoxy resin Epon812 (Durcapan; Fluka AG, Switzerland). Thin sections were cut on LKB-III ultramicrotome and double stained with 2% aqueous uranyl acetate, and lead citrate and then observed with a JEM-100S electron microscope (JEOL).

RESULTS

AmB enhances the isolation rate of primary influenza virus strains in Vero cells. During experiments to isolate influenza viruses from clinical samples, we were surprised to observe that an antibiotic-antimycotic mix had a supportive effect on viral growth in serum-free Vero cell culture. In follow-up experiments we learned that the effect was due to the presence of AmB and that concentrations between 250 and 500 ng/ml had the strongest impact on virus titer (data not shown).

Next, we evaluated the efficiency of virus isolation from clinical specimens using AmB-supplemented Vero cells or MDCK cells as a reference culture. The specimens from several influenza seasons were tested in advance for the presence of a specific influenza virus subtype (H1N1, H3N2, or B or variant H1N1 [vH1N1]). Two consecutive viral passages were carried out in order to detect viral replication by assessing the cytopathic effect and hemagglutination titers. For Vero cells, the presence of AmB dramatically increased the likelihood of virus isolation. The effect was dependent on the influenza virus type and was more prominent for H3N2 and vH1N1 viruses. By adding AmB, 16 of 19 (H3N2) and 9 of 10 (vH1N1) isolates could already be obtained after the first passage of clinical specimens in Vero cells (Table 1). After two passages, isolation rates similar to those of MDCK cells were achieved for all subtypes.

Thus, the often suboptimal sensitivity of Vero cells to infection with primary human influenza viruses could be compensated for by adding AmB into the tissue culture medium. Furthermore, the different influenza virus subtypes showed various degrees of dependence on the effect of AmB.

HA is responsible for the AmB dependence. Next, we investigated the role of surface glycoproteins with respect to virus susceptibility to AmB. Based on the IVR-116 virus backbone, either 6:2 (HA and NA) or 7:1 (HA) reassortant viruses were generated (with AmB) and evaluated for their infectivity and growth in Vero cells in the presence or absence of the drug. First, viruses adjusted to an MOI of 2 were screened for their infectivity by staining infected cells with NP after 18 h of incubation. Figure 1A shows that reassortant viruses containing HA and NA from the human seasonal influenza virus strains displayed reduced infectivity in Vero cells in the absence of AmB. Like the virus isolated from clinical specimens, the vH1N1 virus construct showed the most dramatic drug dependence. In contrast, an analogous reassortant virus with surface glycoproteins of H5N1 A/Vietnam/1203/04 (H5) strain demonstrated the same pattern of infectivity irrespective of the presence of the drug. The NP staining test showed a good correlation with the growth characteristics of the viruses. Figure 1B demonstrates that 6:2 and 7:1 reassortants of A/California/7/2009 (vH1) virus could hardly grow in the absence of

TABLE 1. Enhancement of influenza virus isolation on Vero cells by AmB[a]

Name	Influenza virus strain	Total no. of swabs screened	First passage (no. of swabs)			Second passage (no. of swabs)		
			Vero		MDCK (−AmB)	Vero		MDCK (−AmB)
			−AmB	+AmB		−AmB	+AmB	
H1N1	A/New Caledonia/20/99-like	8	0	0	1	7	8	6
H3N2	A/California/7/2004-like	17	3	14	3	6	16	17
	A/Wyoming/3/2003-like	2	1	2	2	2	2	2
B	B/Malaysia/2506/2004-like	2	2	2	2	2	2	2
	B/Jiangsu/10/2003-like	3	0	1	3	2	3	3
Variant H1N1	A/California/7/2009-like	10	0	9	10	1*	10	10

[a] Subconfluent monolayers of Vero and MDCK cells were inoculated with clinical specimens in the presence (+) or absence (−) of AmB. Cultures were observed over two passages. The presence of virus was assessed by the appearance of the cytopathic effect and then confirmed by the presence of hemagglutination activity. Numbers indicate instances of successful virus isolation. *, A/St. Petersburg/14/2010 isolate.

the drug, whereas analogous constructs containing glycoproteins of A/Vietnam/1203/04 (H5) virus were not drug dependent.

Previously, we demonstrated that the HAs of original human influenza viruses retains its infectivity down to pH values of 5.4, whereas the HAs of H5N1 viruses are not able to infect cells at a pH of ≤5.6 due to an irreversible HA conformational change. It was possible to decrease the threshold of the H5-HA conformational change by introducing a single amino acid substitution into the HA2 subunit at position 58 (K→I) (19). Analogous to the experiments described in Fig. 1A, we tested this virus variant (H5N1-K58I) for its infectivity in the presence or absence of AmB. Unlike the H5N1 virus, the mutated H5N1-K58I virus variant was dependent on AmB (Fig. 1C). This set of experiments demonstrated that the HA plays a major role in defining the virus dependence on AmB and that the drug may promote viral infectivity in Vero cells. Moreover, the AmB dependence might be linked to the pH threshold of the HA conformational change.

AmB promotes the early phase of viral infection. Next, we wanted to elucidate the action mechanism of AmB. In order to investigate which phase of the virus infection is modulated by AmB, we determined the number of infected cells after the addition of AmB 0.5 h prior, simultaneously with and 0.5, 1 and 2 h p.i. Cells were incubated, collected for staining with anti-NP/M-specific antibodies, and analyzed by flow cytometry. A total of 3.5 to 4 times more cells were infected when the cells had been pretreated or when the drug was added simultaneously with or 0.5 h p.i., whereas a later addition of the drug dramatically decreased its effectiveness (Fig. 2). These results indicated that AmB may interact with cellular viral uptake mechanisms such as viral endocytosis and/or uncoating.

To prove this hypothesis, EM was used to visualize virus entry. We infected Vero cells with PR8 H1N1 wild-type virus at an MOI of 30 in the absence or presence of AmB. Thin cell sections were analyzed at 10, 20, 30, and 45 min p.i. At each time point, we screened approximately 80 cells for the presence of virus-loaded endosomes and determined the percentages of the positive cells (Fig. 3A). In the absence of the drug, the number of cells containing virus-loaded endosomes steadily increased to a maximum of 20% at 45 min p.i. Supplementation with AmB resulted in a maximum of 26% of cells containing virus-loaded endosomes already 10 min after infection. At 30 min after infection, the number of virus-loaded endosomes started to decrease progressively in the presence of the drug, which indicates the high extent of viral fusion and uncoating events.

Representative EM images are shown in Fig. 3B. At 10 min p.i. the virus is only attached to the cell surface in the absence of the drug, while virus-loaded endosomes can be observed in the presence of AmB. At 20 min, control cells demonstrate viral uptake by endosomes, whereas frequent fusion events can be registered in the presence of the drug. After 45 min, late endosomes no longer contain virus particles in the presence of AmB, whereas intact virus particles are still detectable within the endosomes in the control culture without AmB.

Thus, in accordance with the previous experiment, our analysis of the EM data revealed that AmB can dramatically accelerate the initial phase of virus infection in Vero cells and most likely also virus endocytosis and viral processing within endosomes.

AmB has a cell-line-specific effect. In order to reveal the cell specificity of AmB action, we used flow cytometry to screen several cell lines for their sensitivity to the drug upon influenza virus infection. In addition to Vero and MDCK cells, A549 (human lung carcinoma), HBE (human bronchial), and primary HNE (human nasal epithelial) cells were infected with a seasonal H1N1 (A/NewCaledonia/20/99-like) virus in the absence or presence of AmB. At 4 h p.i., the cells were detached, fixed, and stained with FITC-labeled α-influenza virus NP/M-specific antibody, and the percentage of influenza virus-infected cells was determined. Addition of AmB to A549, MDCK or HBE cells did not influence the efficiency of infection in these cells. However, the infection efficiency in the AmB-supplemented Vero cells dramatically increased to 88% compared to AmB untreated Vero cells (23%). A similar, obvious increase of infection rate was seen in the HNE cells (74% in AmB-supplemented cells versus 25% in unsupplemented cells) (Fig. 4).

In conclusion, these data demonstrate that the influence of AmB on infection efficiency is cell line specific.

DISCUSSION

AmB is a polyene antibiotic that is widely used for treating serious systemic fungal infections. Under its commercial name, Fungizone, it is also commonly used in animal cell culture to prevent fungal and yeast contaminations. We observed a strong promoting effect of AmB on the recovery rate of influ-

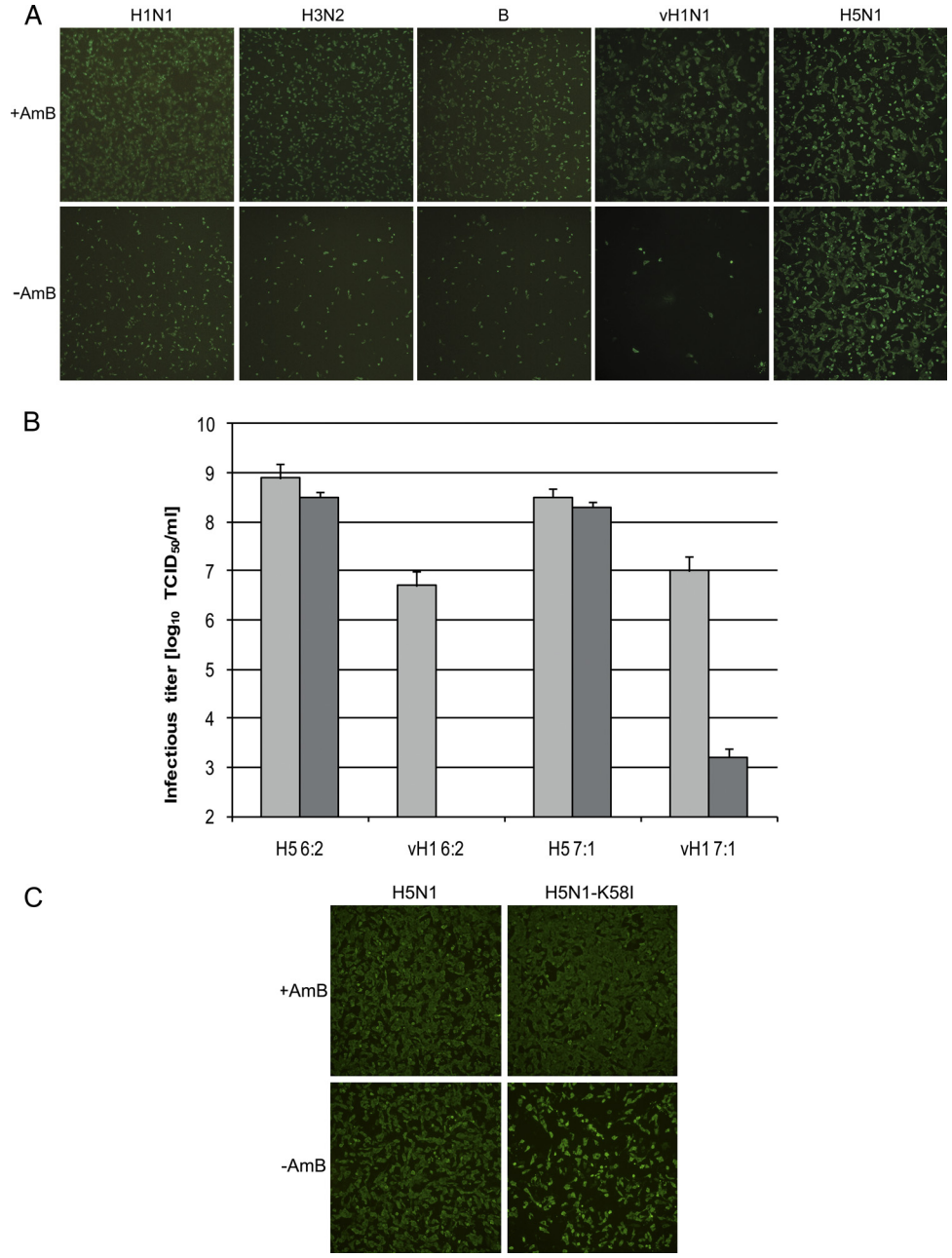

FIG. 1. Influence of AmB on virus infectivity and growth. (A) Virus infectivity. Vero cells were infected with influenza virus H1N1 (A/New Caledonia/20/99-like), H3N2 (A/Brisbane/10/2007-like), B (B/Florida/4/2006-like), vH1N1 (variant A/California/7/2009-like), and H5N1 (A/Vietnam/1203/04-like) ΔNS1 virus constructs (MOI = 2) in the presence (+AmB) or absence (−AmB) of the drug. After 18 h the cells were stained with influenza virus A NP-specific MAb, followed by FITC-conjugated secondary antibody. Pictures were taken at ×10 magnification. (B) Virus growth. Vero cells were infected at an MOI of 0.05 with ΔNS1 virus constructs containing both HA and NA (6:2) or HA only (7:1) from either A/Vietnam/1203/04(H5N1) (H5) or A/California/7/2009(H1N1) (vH1). The remaining virus segments were identical. Cultures were incubated for 48 h in the presence (light columns) or absence (dark columns) of AmB and infectious virus titers ($\log_{10}$ $TCID_{50}$/ml) were determined. Values below the detection limit of the assay (<2 $\log_{10}$ $TCID_{50}$/ml) are not displayed. The means of triplicates (± the standard deviations) are presented. (C) Vero cells were infected with influenza H5N1 (A/Vietnam/1203/04-like) ΔNS1 virus constructs (MOI 5) in the presence (+AmB) or absence (−AmB) of the drug. H5N1-K58I had a single HA2 mutation at position 58 (K→I). Otherwise, the experimental conditions were identical with those described for panel A.

enza virus H1N1, H3N2, and B subtypes during the isolation of influenza virus in Vero cells from clinical samples. We found that a low concentration of AmB (250 ng/ml) increases virus uptake in Vero and human nasal epithelial cells but not in some other cell lines such as MDCK cells. The effect of AmB is related to an early phase of infection. EM images indicate that in Vero cells the process of viral uncoating starts, on average, 20 min earlier in the presence of AmB than in the

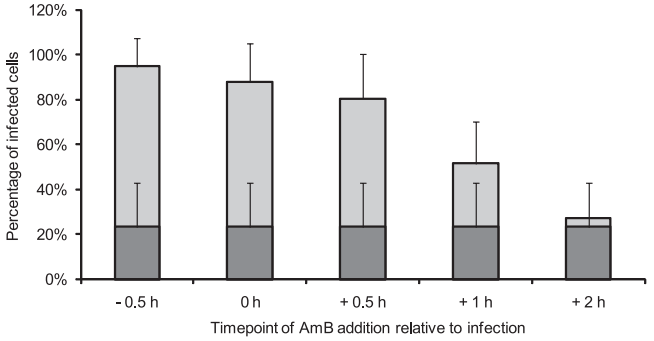

FIG. 2. AmB influences the early phase of viral infection. Vero cells were infected with an influenza A H1N1 (A/New Caledonia/20/99-like) ΔNS1 virus. AmB was added at the indicated time points (light columns). Controls were without AmB (dark columns). At 4 h p.i., the cells were stained with the FITC-labeled anti-NP/M-specific antibodies and analyzed by flow cytometry. The results represent the means (± the standard deviations) of three independent experiments.

group of untreated cells. Different viral strains showed different degrees of dependence on AmB. For example, recent vH1N1 strains could only be isolated in Vero cells in the presence of the drug, whereas seasonal H1N1 viruses from previous years could be obtained more easily without AmB. In contrast to human seasonal strains, several tested H5N1 viruses of highly pathogenic origin and their reassortants could grow to high titers in Vero cells without the drug (19).

In previous experiments we saw that human influenza virus isolates could acquire higher growth ability by accumulating HA mutations after several passages in Vero cells. For example, one of the vH1N1 virus isolates, namely, A/St. Petersburg/14/2010 (HA: GenBank accession no. JF340083) listed in Table 1 became detectable by HA titer after five passages in the absence of the drug. The virus obtained two amino acid substitutions (K119N and T316A) in HA1. While the K119N mutation was shown to influence receptor specificity of the HA (15) and also appeared in the MDCK control culture, the other

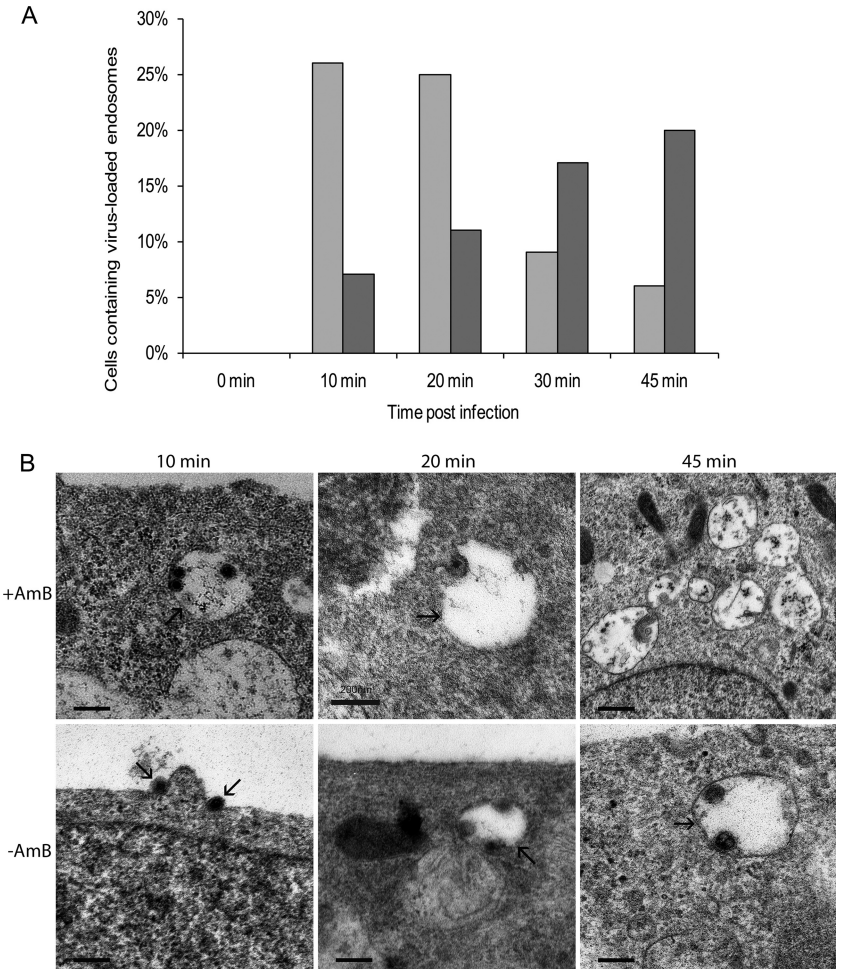

FIG. 3. Endocytic trafficking of influenza virus in the presence or absence of AmB. (A) Vero cells were infected with influenza H1N1 (PR8) wild-type virus in the presence (light columns) or absence (dark columns) of AmB and analyzed by transmission EM at 0, 10, 20, 30, and 45 min p.i. At each time point, an average of 80 cells was screened for the presence of virus-loaded endosomes. The results are shown as the percentage of all cells that contain virus-loaded endosomes. (B) Vero cells were infected with influenza H1N1 (PR8) wild-type virus. After 10, 20, and 45 min of incubation in the presence (+AmB) or absence (−AmB) of AmB, the cells were observed with a JEM-100S electron microscope (JEOL). Black arrows indicate virus-loaded endosomes or virus particles attached to the cell surface (10 min for −AmB only). Black bars indicate lengths of either 200 or 500 nm (45 min for +AmB only).

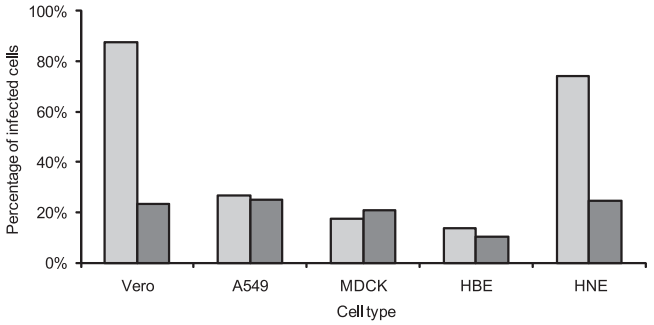

FIG. 4. Effect of AmB on the infectivity of influenza virus in different cell lines. Vero (African green monkey kidney), A549 (human lung carcinoma), MDCK (Madin-Darby canine kidney), HBE (human bronchial), and primary HNE (human nasal epithelial) cells were infected with an influenza A H1N1 (A/New Caledonia/20/99-like) ΔNS1 virus in the absence (dark columns) or presence (light columns) of AmB. At 4 h p.i., the cells were stained with FITC-labeled antibodies against influenza virus NP and M proteins. The percentages of influenza-infected cells were determined by flow cytometry.

mutation (T316A) is located at the carboxy-terminal end of the HA1 subunit, a region which is known to influence the pH value of HA activation (6).

Mutations modulating the fusion properties of the HA (19, 22) were revealed in various Vero cell-adapted viruses of human origin (H1N1, H3N2, and B) and usually went along with a 0.2 to 0.4 higher pH value of fusion (22, 22a). Although supplementation with AmB could not prevent the appearance of HA mutations related to the pH of fusion, its combination with the reduction of the medium pH during infection and cultivation in Vero cells could preserve the original sequences of primary human H3N2 isolates (22). These facts indicate that Vero cells might have an elevated internal pH (pH_i) or defect in their potency to acidify endosomal compartments. An alkaline pH_i for Vero cells was reported elsewhere (31). This might explain the poor growth of original human viruses and the selection of virus variants with an elevated pH level of HA activation in Vero cells. This working hypothesis also fits with previous observations that highly pathogenic H5N1 viruses, which are known for their high values of pH of fusion (19, 29), can grow to high titers in Vero cells without AmB supplementation. At the same time lowering the pH threshold of HA conformational change by introducing the HA2 K58I mutation converted the H5N1 virus into a drug-dependent phenotype (Fig. 1C). Based on these observations, we hypothesize that the action of AmB during influenza virus infection and replication in Vero cells is most likely related to a compensation of an elevated internal and/or endosomal pH.

The exact reasons why Vero cells are unable to support the efficient growth of primary human influenza viruses and the mechanism of AmB compensatory action remain to be elucidated. AmB is known to bind to sterols in cellular membranes. It associates with the ergosterol present in fungal membranes and also, to a lesser extent, with the cholesterols in mammalian cells. This leads to the formation of transmembrane channels (2, 4, 5) and, in turn, to increased permeability to cations. It should be mentioned that these effects were observed at concentrations ranging from 5 to 100 μg of AmB/ml, whereas the experiments described in this publication were carried out at concentrations below 1 μg/ml. Interestingly, Nystatin, another polyene antifungal drug with similar molecular structure, which can also bind to ergosterol and form pores in the membrane, did not induce this virus-growth-enhancing effect in Vero cells (data not shown).

Taking into account all our observations, one possible explanation for the AmB mechanism of action in Vero and HNE cells might be its participation in the modulation of the cellular pH_i by interaction with ATPases (30). It is known that the pH_i of cells and endosomal acidification are mainly regulated by the activity of proton-pumping V-ATPase channels (11). It is also known that the activity of V-ATPase is especially high in epithelial cells of kidney origin (12). Strong surface membrane-associated V-ATPase may keep the pH_i of Vero cells more alkaline than in other cell types and thus create suboptimal conditions for endosomal acidification upon virus infection. Strong external proton pumping could be a similar inherent nature of the nasal epithelial cells due to their ability to provide fast acidification of the respiratory mucosal surface (8). Since AmB was shown to downregulate ATPase activity (30), its addition to Vero or HNE cells may result in the acidification of their internal compartments, which may promote fast pH decrease within endosomes and therefore virus infectivity.

Recently, it was suggested that influenza virus infection triggers the V-ATPase-stimulated endosomal acidification required for virus fusion by the early activation of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase (20). Nothing is known about the effectiveness of these pathways in Vero cells.

To our knowledge, a promoting effect of AmB on virus uptake has not yet been reported. In general, very limited information is available on the effect of AmB and/or its derivatives on virus entry and/or replication. The inhibition of rubella virus growth in rabbit kidney 13 cells by AmB at concentrations of 1 to 5 μg/ml was described previously (33). At a concentration of 12.5 μg/ml, AmB did not inhibit vesicular stomatitis virus when human foreskin fibroblasts were used, whereas AmB methyl ester did (16).

The AmB action mechanism requires additional investigation. Nevertheless, to our knowledge, this is the first evidence of AmB's proviral action. This finding could have a positive impact on the development and production of influenza vaccines in Vero cells.

ACKNOWLEDGMENTS

This study was supported by European Commission grant 518281 FLUVACC.

We are grateful to Monika Redlberger (Institute of Virology, Vienna, Austria), as well as Marina A. Stukova and Michail P. Grudinin (Research Institute of Influenza, St. Petersburg, Russia), for providing clinical isolates and for their support. We also thank Franz Gelhart, Christian Dietzsch, Anita Freudmann, Bernhard Brim, Nicole Ferstl, Bettina Kiefmann, and Bastian Katzler (AVIR Green Hills Biotechnology) for their excellent support in cell culture, virus culture, virus titration, and molecular biology.

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